Biphasic effects of ANP infusion in conscious, euvolumic rats: roles of AQP2 and ENaC trafficking

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1The Water and Salt Research Center, University of Aarhus; 2Institute of Clinical Medicine, Aarhus University Hospital-Skejby, Aarhus; 3Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea; 4Department of Pharmacology, University of Copenhagen, Copenhagen; 5Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; and 6Institute for Basic Psychiatric Research, Department of Biological Psychiatry, Aarhus University Hospital, Aarhus, Denmark

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Wang, Weidong, Chunling Li, Lene N. Nejsum, Hongyan Li, Soo Wan Kim, Tae-Hwan Kwon, Thomas E. N. Jonassen, Mark A. Knepper, Klaus Thomsen, Jørgen Frøkiær, and Søren Nielsen. Biphasic effects of ANP infusion in conscious, euvolumic rats: roles of AQP2 and ENaC trafficking. Am J Physiol Renal Physiol 290: F530–F541, 2006. First published September 20, 2005; doi:10.1152/ajprenal.00070.2005.—Atrial natriuretic peptide (ANP) acutely promotes water and sodium excretion, whereas subchronic doses have been found to have effects on systemic vasculature, cardiac effects, renal vasculature, renal water and electrolyte excretion, and changes in release of a number of different hormones (reviewed in Ref. 6).

The kidney is a major site of action by ANP. By concerted action both on renal vasculature and renal tubular epithelial cells, ANP demonstrates hemodynamic (6) and tubular effects that decrease tubular sodium and water reabsorption; the tubular effects probably predominate vs. the glomerular effects (2, 6, 7, 10). It is well established that ANP activates membrane-bound guanylyl cyclase and modulates cellular functions via the intracellular second messenger cGMP (reviewed in Ref. 6). Studies have provided evidence for stimulation of cGMP production in various renal tubule segments in response to ANP (26, 36) and for the expression of the cGMP-coupled ANP receptor (37) in these segments. ANP administration increases the plasma concentration and urinary excretion of cGMP (10, 18); however, the effect of ANP (through an increase in cGMP) on sodium and fluid transport in the collecting duct is still controversial. Some studies show that it decreases both sodium reabsorption and vasopressin-induced water transport in collecting ducts (14, 27, 28, 31), whereas others do not confirm these inhibitory effects (30, 32).

Moreover, several previous studies have shown that the diuresis and natriuresis induced by ANP are transient when given as continuous infusions or as repeated bolus injections (10, 15, 39) in humans and animals, because resistance to the diuretic and natriuretic actions of ANP develops after relatively brief periods of ANP infusion. A previous study demonstrated that a 4-h ANP infusion in conscious rats resulted in a significant diuresis and natriuresis during the first hour of infusion and then a decline of urine flow rate and urinary sodium excretion to the baseline level, whereas urinary cGMP excretion remained elevated for the duration of the ANP infusion (10). This suggested that either superimposed mechanisms (but not concomitant decline of cGMP) or direct effects of cGMP may blunt the diuretic and natriuretic effects of ANP. The resistance to the diuretic and natriuretic effects of ANP is also
observed in pathophysiological states associated with edema formation, where increased release and elevated plasma level of ANP do not result in diuresis and natriuresis in the degree needed to obtain normal salt and water balance (1, 33). An understanding of the underlying mechanisms that resulted in the resistance to diuretic and natriuretic effects of ANP is becoming important in better understanding the long-term regulation of body water and sodium balance in both physiological and pathophysiological states.

The epithelial sodium channels (ENaC) and aquaporins (AQPs) have been demonstrated to play important roles in renal sodium and water reabsorption in the collecting duct. The apical plasma membrane expression of ENaC subunits (α, β, and γ) and AQP2 can be regulated by changes in the trafficking to the apical plasma membrane. In collecting duct principal cells, AQP2 is shuttled from intracellular vesicles to the apical plasma membrane on vasopressin stimulation; thus collecting duct water permeability, and hence water balance, is acutely regulated by vasopressin-regulated, cAMP-mediated trafficking of AQP2 (reviewed in Ref. 25). The hormone-regulated sodium reabsorption mediated by ENaC is associated with characteristic alterations in the subcellular localization of ENaC subunits (22, 23). Because the natriuretic peptide receptor is expressed in principal cells of the collecting duct (6), and changes in the subcellular localization of AQP2 also involve the activation of the cGMP-dependent pathway (4, 5), we hypothesized that the changes in the subcellular localization of ENaC subunits and AQP2 in the collecting duct principal cells could be involved in the transient phase of natriuresis and water diuresis induced by ANP infusion and in the following phase of decreased ANP effects on natriuresis and diuresis.

The purposes of this study were (1) to reexamine the effects of ANP on renal water and sodium regulation in conscious rats in which sodium and water balance was maintained by infusion of replacement sodium and fluid at a rate to match spontaneous urine losses (servo-controlled replacement) throughout the experiment; (2) to investigate the changes in the subcellular localization of renal collecting duct AQP2, phosphorylated-AQP2 (p-AQP2), and ENaC subunits in response to servo-controlled infusion of ANP at 10 and 90 min after initiation of infusion; and (3) to examine the intracellular AQP2 trafficking in transiently AQP2-transfected HEK 293 cells in response to direct ANP treatment in vitro (in a condition of the absence of vasopressin, angiotensin II, and aldosterone in the culture medium).

MATERIALS AND METHODS

Animals. Specific pathogen-free female Wistar rats (190–220 g) were obtained from M&B (Ry, Denmark). The animals were housed in a temperature (22–24°C)- and humidity (60%)-controlled room with a 12:12-h light-dark cycle. The rats were fed a standard rodent diet (Altromin International, Lage, Germany) containing 120 mmol of sodium and 200 mmol of potassium/kg for at least 10 days before experimentation, with free access to water. For 3 days before the clearance study, lithium citrate was added to the food (15 mmol/kg) to obtain measurable plasma lithium concentration without influencing renal function. The animal protocols have been approved by the boards of the Institute of Anatomy and Institute of Clinical Medicine, University of Aarhus, according to the licenses for use of experimental animals issued by the Danish Ministry of Justice.

Surgical preparation. One week before experimentation, the animals were catheterized. The animals were anesthetized with Hypnorm (0.315 mg/ml fentanyl) citrate + 10 mg/ml fluanisone, 400 μU/kg, Janssen Pharmaceutica, Beere, Belgium)-Dormicum (5 mg/ml midazolam, 800 μU/kg, Hoffmann-La Roche, Basel, Switzerland). With the use of aseptic surgical techniques, sterile Tygon catheters were advanced into the abdominal aorta and the inferior vena cava via the femoral vessels and exteriorized through an incision in the skin of the nape of the neck. A sterile chronic suprapubic bladder catheter was implanted and exteriorized through an incision in the abdominal wall. The arterial and venous catheters were sealed with a 0.9% NaCl subcutaneously (sc; 5 ml) and a long-lasting analgesic (10 μg animal sc, buprenorphinum, Temgesic, SheringPlough, Brussels, Belgium). They were housed individually and, after a recovery period of 5–6 days, they were acclimated to restraining cages to avoid stress during the investigation. The training consisted of three daily sessions with a stepwise increase in duration from 1 to 3 h.

Clearance protocol. The experiments were carried out between 9 AM and 2 PM, with the conscious rats immobilized in restraining cages. The catheters were connected to a Bicore Uniflow blood pressure transducer via the arterial catheter. Through the pressure transducer, a continuous intra-arterial infusion of 25 mM glucose solution containing heparin (100 units/ml) was given at a rate of 5 μl/min to keep the arterial catheter open. Through the venous catheter, the animals received an infusion of 150 mM NaCl solution at a rate of 10 μl/min throughout the experiment (0.6-ml bolus sustained at 10 μl/min) containing [3H]inulin (1.8-μCi bolus, sustained at 0.03 μCi/min, Amersham International, Aylesbury, UK) and LiCl (7.2-μmol bolus sustained at 120 nmol/min). ANP (dissolved in demineralized water; A-8208, Sigma) was given at a dose of 0.5 μg·kg⁻¹·min⁻¹. Controls received vehicle alone. In addition, 25 mM glucose was given intravenously during the initial 120 min at a rate of 30 μl/min to provide an adequate minimum urine flow rate necessary for elimination of bladder-emptying errors. Furthermore, bladders were flushed with demineralized water at a rate of 100 μl/min. Blood samples of 250 μl were collected from the arterial catheter at ~60, 0, 30, and 90 min after the start of ANP or vehicle infusion. All blood samples were replaced immediately with heparinized donor blood.

Servo-controlled system. During the final 90 min of the experiment where ANP was infused, the infusion of 25 mM glucose at a constant rate was stopped and the fluid-sodium balance was maintained by a computer-driven servo-controlled system. From the bladder catheter, urine passed a sodium-sensitive electrode, which performed one measurement of urinary sodium concentration per second (Nova Biochemical, Waltham, MA). Urine was collected every half hour in vials arranged in an autosampler placed on an electronic balance. The autosampler was operated by a photocell, which allowed one to change the vial without touching the balance. Data on urine production (weight on scale) and urinary sodium concentration were sampled continuously on an IBM-compatible computer, which, in turn, controlled the infusion rates of two independent infusion pumps (Harvard model 22, Scandidact, Kvistgaard, Denmark), which delivered 25 mM glucose and 1,200 mM NaCl, respectively. Urinary outputs of sodium and fluid were integrated over the course of 5 min, thus allowing a 5-min delay in changes of sodium and glucose infusion rates.

Analysis and calculations. Urine volume was determined gravimetrically. Plasma sodium, lithium, and urinary sodium concentrations were determined by flame emission photometry. Li⁺ (Li⁺⁺) in the urine was determined by atomic absorption photometry. [3H]Inulin activities in the plasma and urine were determined by liquid scintillation counting on a Packard Tri-Carb liquid scintillation analyzer (Packard Instruments, Meriden, CT). Plasma aldosterone concentrations were determined using a commercially available radioimmunoassay kit (Coat-A-Count, Diagnostic Products, Los Angeles, CA).

Renal clearances (C) and fractional excretions (FE) were calculated by the standard formulas as follows: C = U × V/P; FE = C/GFR, where U is urine concentration, V is the urine flow rate, P is the
plasma concentration, and GFR is the glomerular filtration rate as measured by [3H]inulin clearance.

Cell culture. pcDNA1/Neo with rat cDNA encoding AQP2 (tagged with a COOH-terminal c-myc epitope) was generously provided by Dr. D. Brown (19). HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO2. HEK 293 cells were grown on coverslips coated with poly-D-lysine and transiently transfected using Effectene Transfection Reagent (Qiagen). Cells were kept in serum-free media with indomethacin (10 μM) overnight before the experiments and analyzed 48 h posttransfection. Cells were treated with forskolin (150 μM, Sigma) for 10 min or ANP (10 μM, Sigma) for 10 min. The experiment was repeated three times. Cells were fixed in 4% paraformaldehyde for 10 min, rinsed twice in PBS, and blocked for 15 min in blocking/permeabilization solution (PBS containing 0.1% BSA and 0.1% Triton X-100). Cells were incubated for 1 h at room temperature with primary antibody (see below) diluted in blocking solution, washed three times in PBS, and incubated with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:800, Molecular Probes) for 1 h at room temperature. After three final washes with PBS, the coverslips were mounted with glycagel (DAKO). Cells were analyzed using a Leica TCS-SP2 laser confocal microscope (Heidelberg, Germany).

Immunohistochemistry. Previously characterized rabbit polyclonal antibodies were used: AQP2(24), p-AQP2 (12), and α-, β-, and γ-ENaC subunits (17, 22, 23). For immunoperoxidase labeling, immediately after 10 or 90 min of ANP infusion, rat kidneys were fixed by retrograde perfusion via the aorta [2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4)]. Kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. The paraffin-embedded tissue was cut at 2 μm on a rotary microtome (Leica). The staining was carried out using indirect immunoperoxidase, as previously described (17). In brief, the sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by 0.5% H2O2 in absolute methanol for 10 min at room temperature. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS supplemented with 0.1% BSA and 0.1% Triton X-100. The sections were then washed with PBS followed by incubation in horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (DAKO P448) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. The microscopic examination was carried out with a Leica DMRE light microscope (Leica Microsystems, Herlev, Denmark).

Data presentation and statistics. All values are presented as means ± SE. Individual comparisons within one group were performed by the use of Student’s paired t-test, and individual comparisons between two groups were performed by an unpaired t-test. P values <0.05 were considered statistically significant.

RESULTS

Rapid and transient increase in urine production in response to continuous ANP infusion. Urine production and sodium excretion were monitored in basal condition before ANP infusion (period 1: −60–0 min), in the first phase of ANP infusion (period 2: 0–30 min), and in the later phase of ANP infusion (period 3: 30–90 min, Fig. 1). Infusion of ANP (0.5 μg·kg−1·min−1) resulted in elevations in both urine flow rate and urinary sodium excretion rate (Fig. 1A and B). During the baseline period (period 1), urine flow rate (V) and urinary sodium excretion rate were comparable between the two groups (Fig. 1A and B). In contrast, ANP infusion induced a rapid increase in urine flow rate and peaked 10 min after the initiation of ANP infusion (Fig. 1A, period 2). Subsequently, the urinary flow rate returned to a level comparable to baseline (at 25 min) and maintained the basal level up to 90 min after onset of ANP infusion (Fig. 1A, period 3). Consistent with this, the fractional distal water excretion was significantly reduced (V/CLi, 9.1 ± 0.6 in period 2 vs. 3 ± 0.8% in period 3, P < 0.05, Table 1) in ANP-treated rats, suggesting that renal water reabsorption was increased in the collecting ducts in period 3 compared with period 2 despite continued infusion of ANP. In time-matched controls, the urine flow rate did not change during the whole experimental period (Fig. 1A).

Increased sodium excretion in response to ANP continuous infusion. The urinary sodium excretion rate increased significantly and peaked 15 min after the onset of ANP infusion and declined gradually after the peak but remained elevated compared with time-matched controls (Fig. 1B, periods 2 and 3). Thus in contrast to the effects of ANP on urine flow rate, the sodium excretion rate was maintained significantly higher throughout the 90-min infusion (Fig. 1B). Consistent with this, the fractional sodium excretion (FENa; 3.2 ± 0.3 vs. 0.4 ± 0.1%, Table 1) and fractional distal sodium excretion (CNa/CLi; 6.8 ± 1.1 vs. 1.1 ± 0.2%, Table 1) were significantly increased in period 2 compared with period 1. Similarly, significant differences were also observed in FEK+ (1.7 ± 0.6 vs. 0.4 ± 0.1%, Table 1) and K+ fractional excretion (Ck+/CLi; 4.1 ± 0.8 vs. 1.1 ± 0.2%, Table 1) in period 3 after ANP infusion compared with baseline period 1. Control rats did not show any changes in sodium excretion during the whole experiment (Table 1).

Fig. 1. Time course of values for urinary flow rate (A) and urinary sodium excretion (B). Control rats: ■, atrial natriuretic peptide (ANP)-infused rats. ANP infusion starts in the treated animals at time 0. Period 1, −60–0 min; period 2, 0–30 min; period 3, 30–90 min. *P < 0.05 compared with control rats.
Absence of changes in proximal water/sodium reabsorption or in GFR in response to continuous ANP infusion. During periods 2 and 3, proximal tubular fluid output (V\text{prox}, Cl\text{li}) and fractional lithium excretion (FE\text{Li}) did not change in any of the two groups (Table 1), indicating that ANP had little or no effect on proximal tubule water and sodium reabsorption. Similarly, GFR (C\text{in}) did not change during ANP infusion (Table 1). In ANP-infused rats, mean arterial blood pressure (MAP) fell within 10 min following the start of ANP infusion and reached a stably low level throughout the infusion period. In control rats, MAP did not change throughout the whole experimental period. The difference in MAP between periods 1 and 3 was significant in ANP-infused rats (20 ± 3 vs. 6.8 ± 1 mmHg in control rats, P < 0.05, n = 6/group). Plasma electrolyte concentrations were similar before and during treatment with ANP (data not shown).

Absence of significant changes in apical labeling of AQP2 and p-AQP2 in IMCD principal cells in the initial phase of ANP infusion (period 2). Immunohistochemistry revealed no significant changes in the subcellular labeling of both AQP2 and p-AQP2 [using an antibody that selectively labels AQP2 phosphorylated in the PKA phosphorylation consensus site (Ser 256)] in the inner medullary collecting duct (IMCD) cells between controls and ANP-infused animals after 10 min of ANP infusion (Fig. 2, A–D).

Increased apical labeling of AQP2 and p-AQP2 in IMCD principal cells in response to 90 min of ANP infusion (period 3). We next tested the effect of ANP infusion on AQP2 and p-AQP2 expression in IMCD principal cells by immunohistochemistry in the late phase of ANP infusion (period 3). In control rats, immunoperoxidase microscopy revealed labeling of apical plasma membrane domains in all levels of the

![Image of immunoperoxidase microscopy of AQP2 and p-AQP2](image-url)
collecting duct. In the cortical collecting duct (CCD) and outer medullary collecting duct (OMCD), AQP2 was mainly associated with the apical plasma membrane and subapical domains (Fig. 3, A and C). In IMCD principal cells, AQP2 labeling was seen in both apical and basolateral plasma membrane domains as well as in intracellular domains (Fig. 3, E and G). In ANP-infused rats, AQP2 labeling in the CCD was mainly associated with the apical plasma membrane and subapical domains similar to the AQP2 labeling in control rats (Fig. 3B). In the inner stripe of outer medulla (ISOM), some principal cells exhibited increased apical labeling of AQP2 (arrow in Fig. 3D); however, most of the principal cells in the ISOM did not show different labeling distribution of AQP2 (not shown).

In contrast, IMCD principal cells exhibited prominent apical labeling of AQP2 and intracellular labeling of AQP2 is decreased (arrow in Fig. 3H). Magnification: ×630 (A–D, G, and H); ×250 (E and F).

Fig. 3. Immunoperoxidase microscopy of AQP2 in the cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and IMCD from CON rats (A, C, E, and G) and rats infused with ANP for 90 min (B, D, F, and H). In the control rat, AQP2 is localized mainly in apical plasma membrane domains of CCD principal cells (A), whereas intercalated cells are not labeled (arrowhead). In OMCD principal cells, the immunolabeling of AQP2 is mainly associated with the apical plasma membrane and intracellular vesicles (C). In IMCD principal cells, AQP2 labeling is seen in both apical plasma membrane domains and intracellular domains (E and G). In the ANP-infused rats, similar labeling of AQP2 is seen in CCD principal cells (arrows in B), whereas some OMCD principal cells in the inner stripe of outer medulla show distinct apical labeling of AQP2 (arrow in D) with little intracellular labeling, whereas no intercalated cells are labeled (arrowhead in D). In contrast, IMCD principal cells exhibit prominent apical labeling of AQP2 and intracellular labeling of AQP2 is decreased (arrow in H). Magnification: ×630 (A–D, G, and H); ×250 (E and F).
labeling were observed in cortical (Fig. 4B) and outer medullary collecting duct principal cells (not shown) in response to 90 min of ANP infusion.

**ANP stimulates AQP2 translocation in HEK 293 cells transiently transfected with AQP2.** To confirm the in vivo results in a condition lacking vasopressin, angiotensin II, and aldosterone, all of which have been demonstrated to have effects on AQP2 regulation, we tested the direct effect of ANP treatment on the subcellular AQP2 localization in HEK 293 cells, which were transiently transfected with AQP2. In nontreated HEK 293 cells, AQP2 was exclusively localized in intracellular vesicles (arrowheads in Fig. 5A). In contrast, in response to forskolin, an activator of cAMP production, AQP2 was translocated to the plasma membrane (arrows in Fig. 5B). Stimulation with ANP also resulted in translocation of AQP2 to the plasma membrane (arrows in Fig. 5C), supporting the results obtained in vivo in the present study.

**Increased apical labeling of α- and γ-ENaC in CCD principal cells in response to prolonged ANP infusion.** To investigate whether the intracellular trafficking of ENaC subunits is altered in ANP-infused rats, we carried out immunoperoxidase microscopy of α-, β-, and γ-subunits. Immunolabeling confirmed previous studies showing ENaC expression from the second half of the distal convoluted tubules (DCT2) to the IMCD, with the expression being most prominent in DCT2, connecting tubules (CNT), and CCD, with decreased labeling in medullary collecting duct segments (17). In control rats, immunoperoxidase microscopy demonstrated that immunolabeling of α (Fig. 6)-, β (Fig. 7)-, and γ (Fig. 8)-ENaC subunits was exclusively associated with the apical plasma membrane and the intracellular vesicles in DCT2, CNT, and CCD, consistent with previous findings (17) (data not shown for medullary collecting duct). After 10 min of ANP infusion, no significant changes were observed in the subcellular local-

![Fig. 4. Immunoperoxidase microscopy of p-AQP2 in CCD and IMCD from CON rats (A and C) and rats infused with ANP 90 min (B and D). In the CON rats, p-AQP2 is localized mainly in apical plasma membrane domains of CCD principal cells (A), whereas intercalated cells were not labeled (arrowhead). In IMCD principal cells, p-AQP2 labeling is seen in both apical plasma membrane domains and intracellular domains (C). In the ANP-infused rats, similar labeling of p-AQP2 was seen in CCD principal cells (B), whereas IMCD principal cells exhibited prominent apical labeling and less intracellular labeling (arrow in D). Magnification: ×630.](http://ajprenal.physiology.org/)

![Fig. 5. Laser-scanning confocal immunofluorescence microscopy of AQP2 in HEK 293 cells transiently transfected with AQP2. In nonstimulated cells (control), AQP2 is mainly localized to intracellular vesicles (arrowheads in A). On stimulation with forskolin, AQP2 is translocated to the plasma membrane (arrows in B). Stimulation with ANP also induces translocation of AQP2 to the plasma membrane (arrows in C). Bar = 10 μm.](http://ajprenal.physiology.org/)
ization of ENaC subunits (not shown). In contrast, 90-min ANP infusion induced a marked increase in apical labeling of \( \beta \)-ENaC and \( \gamma \)-ENaC in DCT2, CNT, and CCD principal cells (Fig. 6, B, D, and F, and Fig. 8, B, D, and F, respectively) compared with control rats (Fig. 6, A, C, and E, and Fig. 8, A, C, and E, respectively). No changes were observed in the IMCD (not shown). In contrast, the labeling of \( \beta \)-ENaC was unchanged and showed both apical plasma membrane and dispersed cytoplasmic labeling in DCT2, CNT, CCD, and IMCD cells in both control (Fig. 7, A, C, and E) and ANP-infused rats (Fig. 7, B, D, and F). Therefore, the increased apical ENaC expression may suggest a possible compensation for the natriuresis induced by the continuous infusion of ANP. On the other hand, plasma aldosterone levels at period 3 did not show any significant difference between the two groups (1,608 ± 370 in ANP-infused rats vs. 1,565 ± 530 pg/ml in controls; not significant). These findings suggest that plasma aldosterone may not play a role in the increased apical targeting of ENaC subunits in rats with ANP infusion.

**DISCUSSION**

The present study was performed to examine the effects of ANP on renal sodium and water metabolism and on the regulation of intracellular AQP2 and ENaC trafficking. The overall purpose was to examine whether changes in subcellular localization of AQP2 and ENaC subunits could explain the marked changes in the water and sodium metabolism seen in response to acute (10 min) and subacute (90 min) ANP treatment, with the initial phase promoting diuresis and the later
phase revealing a resistance to diuretic effect of ANP. ANP infusion results in a rapid, transient diuresis and a persistent increase in urinary sodium excretion with no changes in GFR and no changes in the subcellular localization of AQP2 and ENaC subunits in the initial phase. This indicates that changes in AQP2 and ENaC subunits do not play a major role in ANP-induced diuresis and natriuresis in the initial phase. In contrast, ANP infusion for 90 min was associated with an increased translocation of AQP2 and ENaC to the apical plasma membrane in the principal cells of collecting ducts, suggesting a compensatory process in response to persistently increased urinary sodium excretion induced by ANP.

Anesthesia and changes in body fluid homeostasis are known to influence the circulating concentrations of hormones, including vasopressin and ANP (7, 40). To exclude the confounding influence of anesthesia and disturbances in body fluid status, which are often seen in experimental animals on fixed-infusion regimens, the present experiments were conducted in chronically catheterized, conscious rats using the servo-controlled, fluid-balanced, fluid-replacement technique to maintain the extracellular fluid volume in these animals.

ANP-induced diuresis and natriuresis and its resistance. In the present study, continuous ANP infusion induced diuresis and natriuresis, consistent with previous studies in animals and humans (2, 7, 8, 10, 39). The effect of ANP (lowering of blood pressure) was persistent throughout the study. In contrast, GFR was slightly, but insignificantly increased during periods when diuresis and natriuresis were highest (period 2), although a transient change in GFR at a certain time point cannot be excluded. Unchanged GFR in response to ANP infusion was consistent with previous studies in which the dosages of ANP were one-half or two-thirds that of the dose used in this study (2, 7, 10). It is likely that systemic ANP infusion does not alter the GFR level despite a significant decrease in systemic blood pressure. The fact that urinary sodium excretion was elevated despite a fall in blood pressure supports the idea that ANP acts on the kidney to shift the so-called pressure-natriuresis curve. Persistent reduction of MAP was also seen in the other servo-controlled study (2). Mechanisms by which ANP reduce blood pressure include diminished cardiac output, reductions in peripheral vascular resistance, vasodilation, and decreased intravascular volume, volume status, and circulating hormone (6). Maintenance of extracellular fluid by a servo-controlled system may not fully prevent the hypotensive effect of ANP.

The observed occurrence of diuresis and natriuresis without a detectable change in GFR in response to ANP infusion would...
be compatible with the hypothesis that ANP also directly alters renal tubule sodium and water reabsorption. In the present study, the proximal tubular fluid output (CLi) and FELi were not changed by the infusion of ANP, indicating that ANP-induced natriuresis is associated with the decreased sodium reabsorption in the distal nephron and collecting duct, but not in the proximal tubule. The loss of water and sodium after ANP infusion was immediately replaced by the servo-controlled system, and this gave us the opportunity to examine the effect of ANP on sodium and water excretion when the renal effect of ANP was unopposed by sodium and water losses. Surprisingly, after an initial peak in urine flow rate, it went down to the baseline level. In contrast, sodium excretion, after an initial peak, remained elevated by fivefold compared with the pretreatment baseline level. In our studies, resistance or blunted effects to the diuretic effect of ANP, was associated with enhanced apical labeling of AQP2 and p-AQP2 in IMCD principal cells. This finding was confirmed by our in vitro studies, which showed that ANP induced plasma membrane insertion of AQP2 in renal epithelial cells (4, 5), the present data further suggest a cGMP-dependent pathway for AQP2 membrane insertion in renal epithelial cells, in addition to a cAMP-dependent pathway.

AQP2 is the primary target for vasopressin regulation of collecting duct water permeability and body water balance. The most widely understood pathway leading to AQP2 membrane targeting is via vasopressin V₂ receptor-mediated stimulation of adenylyl cyclase, cAMP-mediated activation of protein kinase A, and phosphorylation of AQP2. AQP2 is then translocated from intracellular vesicles to the plasma membrane, thereby increasing the water permeability of the apical plasma membrane (reviewed in Ref. 25). These studies em-

**Fig. 8.** Immunoperoxidase microscopy of γ-ENaC in DCT2, CNT, and CCD from CON rats (A, C, and E) and rats infused with ANP for 90 min (B, D, and F). Immunoperoxidase labeling of γ-ENaC is mainly observed to be dispersed in the cytoplasm of principal cells in the DCT2 (A), CNT (C), and CCD (E) in CON rats. In contrast, γ-ENaC labeling is predominantly localized to the apical plasma membrane domains in DCT2 (B), CNT (D), and CCD (F) principal cells of ANP-infused rats. Arrows indicate apical labeling of γ-ENaC, whereas arrowheads indicate intercalated cells. Magnification: ×1,000.
phasized the important roles of cAMP in mediating AQP2 trafficking under vasopressin regulation. In addition, recent studies demonstrated a cAMP-independent and cGMP-dependent pathway for AQP2 membrane insertion in renal epithelial cells (4, 5). In these studies, exogenous cGMP or increased endogenous cGMP levels induced by sodium nitroprusside and ANP treatment stimulated relocation of AQP2 from cytoplasmic vesicles to the plasma membrane in rat kidney collecting duct principal cells and LLC-PK1 cells stably transfected with AQP2 (4). The observed AQP2 membrane insertion by elevated intracellular cGMP could be achieved by inhibition of cGMP phosphodiesterases (5). Consistent with these findings, the present data showed a strong plasma membrane staining for AQP2 in HEK cells transfected with AQP2 exposed to ANP. Moreover, importantly, we demonstrate a strong apical plasma membrane labeling of AQP2 and p-AQP2 in IMCD principal cells of rats infused with ANP. It should be noted that it is unclear whether the AQP2 trafficking is a direct effect of cGMP and ANP or a secondary effect.

The selective effect of ANP to induce AQP2 plasma trafficking changes in IMCD remains unclear. However, this is consistent with recent evidence of a cGMP-mediated (by ANP and inhibition of cGMP phosphodiesterases) increase in AQP2 accumulation in the apical plasma membrane of principal cells mainly in the medullary collecting ducts (4, 5), suggesting that medullary collecting duct principal cells appears to be more sensitive to ANP (or cGMP) than CCD for AQP2 translocation. Further studies are needed to clarify the detailed cellular mechanisms of ANP-induced AQP2 regulation.

ANP induced translocation of α- and γ-ENaC subunits to the plasma membrane. Consistent with previous studies, ANP infusion led to an acute increase in sodium excretion. The greatest natriuretic effect was seen 15 min after infusion, and then the urinary sodium excretion went down to a plateau that was about five times higher than the baseline level. FENa and CNa/CLi at the plateau were also lower than the levels observed at the peak, suggesting the existence of a compensatory effect in the distal nephron and the collecting duct. The changes in urinary sodium excretion were associated with increased apical labeling of α- and γ-ENaC subunits in the DCT2, CNT, and CCD. Thus it is believed that the reabsorption of sodium through the apical membrane by α- and γ-ENaC in these renal tubular segments compensates for increased sodium excretion during the peak time.
The apical plasma membrane expression of ENaC can be altered by changes in the trafficking of the channel subunits to the apical plasma membrane under hormone regulation. These studies suggested that translocation of ENaC to the apical plasma membrane is strictly regulated by several hormones, such as aldosterone (22, 23), vasopressin (20), and angiotensin II (3). However, the regulatory mechanisms involved in the altered apical plasma membrane expression of ENaC seen in response to ANP are not clear. It may represent direct effects of ANP or potentially also involve secondary effects by other hormonal systems. Two previous studies using similar techniques have shown unchanged plasma vasopressin levels during ANP infusion (2, 7). Thus it appears unlikely that AVP is involved in the translocation of ENaC subunits to the apical plasma membrane. ANP is known to directly inhibit adrenal aldosterone (11) and renin (9) secretion and to be indirectly involved in the regulation of sodium reabsorption through suppressing the renin-angiotensin-aldosterone axis. In the two studies on euvoletic rats infused with ANP, aldosterone levels were reduced in one study (7), but maintained in the other (2). Aldosterone levels did not change in response to ANP infusion in the present study, indicating other factors rather than hormones may regulate apical expression of ENaC subunits. Whether intrarenal angiotensin II levels may be increased in ANP-infused rats cannot be ascertained from the present data.

In the present study, ANP infusion induced significant natriuresis. However, apical targeting of ENaC subunits was markedly increased, which plays a role in sodium reabsorption in the distal nephron and collecting duct. Thus a straightforward interpretation of these results would be that lack of downregulation of ENaC expression at the site of function, the apical plasma membrane, in the distal nephron speaks against changes in ENaC subunits playing a role in ANP-induced natriuresis and diuresis. Besides ENaC, an amiloride-sensitive, cGMP-gated cation channel located in the apical membrane of kidney cells is revealed to mediate transepithelial sodium reabsorption across the distal nephron and the collecting ducts (21). Because the rates of electrogenic, amiloride-sensitive sodium absorption in the CCD and IMCD are similar (29, 34, 38), the observation that ENaC expression is lower in IMCD than in CCD suggests that cholesterol, such as the cGMP-gated cation channel, along with ENaC participates in electrogenic sodium absorption across the IMCD. ANP (via cGMP) has been demonstrated to reduce electrogenic sodium absorption across the IMCD by inhibiting the cGMP-gated cation channel (21, 35). By functioning independently and/or by being regulated independently, two sodium-conducting channels may impart more diverse and precise control over sodium reabsorption, which is essential for the regulation and maintenance of extracellular fluid volume.

The present study raised a possible explanation for the resistance to diuresis and natriuresis of ANP during prolonged ANP infusion seen in other studies (10, 39) and some chronic edematous states in which positive sodium balance persists despite elevated plasma levels of ANP (1, 33). In these studies, prolonged ANP infusion induced a transient diuresis and natriuresis despite persistently increased urinary cGMP excretion in rats. In patients with heart failure, ANP levels are elevated and a linear relationship between plasma ANP and urinary cGMP is observed (1). These data indicated a potential role of cGMP in mediating ANP’s effects. ANP-induced, cGMP-mediated apical plasma membrane expression of AQP2, α-AQP2, and α- and γ-ENaC may promote water and sodium reabsorption in the distal nephron and the collecting ducts and may be involved, at least in part, in the evanescence of diuresis/natriuresis and water and sodium retention in some conditions. However, further studies are warranted to examine the mechanisms for the development of ANP resistance.

In summary, our study demonstrated that continuous infusion of ANP evoked a transient, significant diuresis and sustained natriuresis in conscious, euvoletic rats. ANP administration was associated with strong apical plasma membrane labeling of AQP2 and α-AQP2 in the IMCD principal cells and plasma membrane insertion of AQP2 in HEK cells transiently transfected with AQP2. It is likely that ANP via cGMP induces an intracellular redistribution of AQP2. Prolonged ANP stimulation was also associated with enhanced apical plasma membrane location of α- and γ-ENaC in the CCD, potentially indicating a local regulation of ENaC translocation in the kidney in response to increased urinary sodium excretion induced by ANP.

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